

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 51/00, 38/00, C07K 2/00, 5/00, 7/00, C07F 5/00		A1	(11) International Publication Number: WO 98/52618 (43) International Publication Date: 26 November 1998 (26.11.98)
(21) International Application Number: PCT/US98/10186 (22) International Filing Date: 19 May 1998 (19.05.98)		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 08/858,971 20 May 1997 (20.05.97) US		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/858,971 (CIP) Filed on 20 May 1997 (20.05.97)			
(71) Applicant (<i>for all designated States except US</i>): THOMAS JEFFERSON UNIVERSITY [US/US]; 11th and Walnut Streets, Philadelphia, PA 19107 (US).			
(72) Inventors; and (75) Inventors/Applicants (<i>for US only</i>): THAKUR, Mathew, L. [US/US]; 44 Lakeview Drive, Cherry Hill, NJ 08003 (US). PALLELA, Venkataramana, R. [IN/IN]; 49B Marion Court, Upper Darby, PA 19082 (US).			
(74) Agents: LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, 66 E. Main Street, Marlton, NJ 08053 (US).			

(54) Title: RADIOLABELED THROMBUS IMAGING AGENTS

(57) Abstract

This invention relates to a radiodiagnostic agent to image arterial and venous thrombi, pulmonary emboli and lesions of atherosclerosis. A composition for a thrombus imaging agent, a method and kit for preparing a thrombus imaging agent, and a radiolabeling reagent for preparing the thrombus imaging agent are provided. Methods of using the thrombus imaging agent to detect thrombi are also provided.

AN3

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		

RADIOLABELED THROMBUS IMAGING AGENTS**FIELD OF THE INVENTION**

This invention relates to radiolabeled agents for imaging thrombi, methods of use of said agents, and kits for preparing imaging agents.

BACKGROUND OF THE INVENTION

In 1993, in the United States alone, 378,000 patients were hospitalized for deep venous thrombosis (DVT) and 103,000 for pulmonary embolism (PE) (Vital and Health Statistics. Series 13: Data from National Health Survey Ditts Publication No. (PHS) 95-1783, 1993). Autopsy data suggests that the incidence of DVT and PE may be as high as 30% to 60% in the general population. For high risk patients, such as those over forty years of age or those who have undergone a major surgical procedure, the incidence of thromboembolism may be as high as 80%. (Koblik, P.D. et al., "Current status of immunoscintigraphy in the detection of thrombosis and thromboembolism", *Seminars in Nucl. Med.* XIX:221-231, 1989)

In surgical patients, the initial vessel damage exposes subendothelial structures to the blood stream and the blood platelets begin to adhere to the injury site. Coagulation proteins are then activated sequentially to generate the enzyme thrombin. Thrombin cleaves plasma fibrinogen into fibrin monomers, which in turn polymerize around the clumped platelets to form a clot. Blood clots are also formed during stasis in patients confined to bed, or in those with increased

- 2 -

intra-abdominal pressure (e.g., during pregnancy, when blood flow slows in the leg veins). Under these hemodynamic conditions, especially near venous valves or bifurcations, the coagulation factors are more likely to be activated. Many of 5 the clots formed under these conditions are likely to be large and frequently form lethal pulmonary emboli. Arterial clots are considered to be the leading cause in the pathogenesis of myocardial ischemia or infarction and stroke.

A variety of diagnostic tests are currently available 10 to detect DVT. These include contrast angiography (venography), radionuclide angiography, Doppler ultrasonography, thermography and impedance plethysmography. Most popular among them are the venography and ultrasonography. However, to be effective, venography and 15 ultrasonography must be performed in the specific anatomic area of the suspected emboli. Notwithstanding this limitation, contrast venography is widely considered as the gold standard for the diagnosis of thromboembolism. Additionally, venography is an invasive technique, and the 20 procedure itself can induce venous thrombi in patients at risk. Anticoagulant therapy to prevent formation of such venous thrombi or to lyse the existing DVT is also associated with a significant morbidity. For these reasons, a more specific and non-invasive method for imaging thrombi in the 25 body is highly desirable. An external scintigraphy technique, aided by the use of a radiopharmaceutical, would enable a clinician to quickly scan a patient without unreasonable inconvenience or added morbidity.

In recent years, a large number of radiopharmaceuticals 30 have been investigated as potential agents to localize DVT or PE. In that thrombi are largely composed of fibrin, platelets and other entrapped cells in the fibrin network, attention has been focused on the use of radioiodine labeled fibrinogen and In-111 labeled platelets (Thakur, M. L. et al. *Thrombosis Research* 1976, 9, 345-354). Antibodies specific for the 35 fibrin binding IIb/IIIa glycoprotein complex on the platelet surface have also been investigated (Thakur, M.L. *Thrombotic*

- 3 -

and *Hematologic Disorders* 1992, 5, 29-36; Thakur, M.L. "Radiolabeled monoclonal antibodies for imaging and therapy", S.C. Srivastava (Ed.), Plenum Publishing Co., NATO ASI, series 152, 1988; Knight, L.C. *Seminars in Nucl. Med.* 1990, XX:52-67.

- 5 Success of these approaches has been limited due to a lack of specificity, unfavorable pharmacokinetics or cumbersome preparation of the agent.

In that platelets are a major component and a biologically active constituent of a thrombus, radiolabeling 10 of platelets has also been considered as a diagnostic agent. However, the use of radiolabeled platelets has been less than desirable because of their long life span (roughly 8 days) that results in excessive background radioactivity well after their administration. The excessive background radioactivity 15 causes a delay in diagnosis due to suboptimal lesion to background radioactivity ratios. Use of radiolabeled platelets is also limited by the need to prepare them *in vitro* by skilled personnel (Thakur, M. L., 1976, *supra*).

Prompted by advancements in molecular biology, 20 radioactive agents for the non-invasive diagnosis of thromboembolism have been developed which include technetium (Tc-99m) labeled peptides specific for resting or activated platelets (Knight, L.C. et al. *J. Nucl. Med.* 1994, 35, 282-288; Pearson, D.A. *J. Med. Chem.* 1996, 39, 1372-1382; 25 Line, B.R. et al. *J. Nucl. Med.* 1996, 37, 117P). Use of a peptide specifically binding to the platelet GPIIb/IIIa receptor has also been reported for imaging thrombi (Dean, R. et al., WO/94/23758).

Peptides are particularly attractive for use as 30 radioimaging agents because they are smaller in size and easier to produce than monoclonal antibodies. Radiolabeled peptides typically clear more rapidly from circulation than radiolabeled proteins, are less likely to induce an immunological reaction, yet have equal or higher receptor 35 specificity and binding constants than monoclonal antibodies. While a variety of radioactive nuclides have been considered for radiolabeling peptides, including Tc-99m and In-111,

- 4 -

Tc-99m is a preferred radiolabel because of its low cost, availability, excellent imaging properties (emits gamma radiation at 140 keV), and high specific activity. Its half-life of 6 hours is long enough to perform an examination 5 before excessive radioactive decay has occurred, yet not so long as to persist in the body long after the examination has been completed or to impart an excessive radiation dose to the patient.

Radiopharmaceuticals comprised of radiolabeled peptides 10 and proteins can be prepared by three routes. One such route includes the use of a chelating agent such as that disclosed in U.S. Patent No. 5,552,525. A second method utilizes a bifunctional chelating agents, such as that disclosed in U.S. Patent No. 5,218,128. Typically, the third method, known as 15 a direct labeling method, has been used for labeling antibodies and peptides and involves the generation of free sulphydryl groups by the reduction of disulfide bridges to which the radionuclide, such as technetium, is chelated. See, for example, U.S. Patent Nos. 4,917,878 and 5,011,676. The 20 direct labeling method suffers from two prominent limitations, namely, the unstable complexes formed by technetium with the peptide or protein, and the poor control over the labeling site with the protein. It is not likely that direct labeling can be readily extended for use with small peptides because 25 small peptides may not contain cysteine residues that are cyclized and the biological function of a small peptide may be more often altered by random addition of the radiolabel.

Bifunctional chelates have been developed which utilize 30 high affinity chelates to bind a technetium radionuclide to specific sites on the peptide. In this latter method the chelating agent is covalently attached to the peptide prior to radiolabeling, which upon the addition of the radionuclide would result in a single radiolabeled product. This approach appears to overcome some of the limitations of the direct 35 method of labeling in that the bioactivity and receptor-binding characteristics of the conjugate can be determined before and after labeling.

- 5 -

Thrombospondin (TSP), is a large (450 Kda), trimeric adhesive glycoprotein first identified as a thrombin sensitive protein more than a quarter of a century ago (Baenziger, N.L. et al. *Proc. Natl. Acad. Sci. USA* 1971, 68, 240-243; Ganguly, 5 *P. J. Biol. Chem.* 1971, 246, 4286-4290). TSP is predominantly found in platelet α -granules and comprises nearly 3% of the total amount of platelet protein. Many different cells produce TSP, including endothelial cells, fibroblasts, macrophages and monocytes, and tumor cells. It is known that 10 most of TSP is bound to extracellular matrix or associated strongly with basement membrane. TSP promotes cell-cell and cell-matrix interactions of normal and malignant cells, as well as platelet aggregation, and mediates angiogenesis.

TSP is stored in resting platelets in α -granules 15 alongside other multi-functional glycoproteins such as fibronectin, fibrinogen and von Willebrand's factor. Upon activation, platelets release α -granule proteins including TSP, which then binds to the surface of activated platelets. Receptors for TSP binding on platelet surface include CD36, 20 a 88 kd glycoprotein, GPIV and GPIIb. One theory proposes that TSP binds to CD36 as well as to fibrinogen that is already bound to GP IIb/IIIa complex. TSP is found in fibrin meshwork of whole blood clots and excised wounds. Early wounds stain intensely for TSP whereas healed wounds hardly 25 stain.

The structure of human TSP was determined in 1986 (Lawler et al. *Blood* 1986, 67, 1197-1209). It was found that the sequence Trp Ser Pro Cys Ser Val Thr Cys Gly (SEQ ID NO: 1) was present in three homologous copies in TSP, and that the 30 sequence is a functional component in the adhesive interactions of TSP that mediate cell adhesion, platelet aggregation and tumor cell metastasis (Tuszynski, G.P. et al. *J. Cell Biol.* 1992, 116, 209-217).

It has been reported that human dermal microvascular 35 endothelial cells bind to immobilized TSP via an Arg Gly Asp Cys Ser Val Thr Cys Gly sequence (SEQ ID NO: 2) (Chen et al. *J. Invt. Dermatol.* 1996, 106, 215-220), and that a

- 6 -

radioiodinated (I-125) Tyr Cys Ser Val Thr Cys Gly sequence (SEQ ID NO: 3) also binds strongly to CD36 transfected Jurkat cells (Asch et al. *Biochem. Biophys. Res. Comm.* 1992, 182, 1208-1217). It has also been demonstrated that antibodies 5 against TSP receptor blocked the uptake of TSP as well as that of the sequence, Cys Ser Val Thr Cys Gly (SEQ ID NO: 4) (Wang et al. *Am. J. Surg.* 1995, 170, 502-505).

Derivatives of the sequence Cys Ser Val Thr Cys Gly (SEQ ID NO: 4), have also been shown to have biological activity. 10 For example, a cyclic form in which two cysteine residues are cyclized to form a five membered ring structure, and a derivative in which the two cysteine residues are blocked by acetaminomethyl (AcM), promoted binding to four tumor cell lines, approximately to the same extent as the native sequence 15 (SEQ ID NO: 4) (Tuszynski et al. *J. Cell. Biol.* 1992, 116, 209-217). The native sequence (SEQ ID NO: 4), as well as the cyclic and cysteine protected forms, inhibited ADP-induced platelet aggregation, while control peptides, Ala Asn Lys His 20 Tyr Phe (SEQ ID NO: 5) and Val Cys Thr Gly Ser Cys (SEQ ID NO: 6) displayed no biological activity.

It has now been found that a modification of the peptide sequence, Cys Ser Val Thr Cys Gly (SEQ ID NO: 4) can be used to image arterial and venous thrombi, pulmonary embolisms, and lesions of atherosclerosis. Unlike other peptides currently 25 being investigated for use in imaging thrombi, such as, snake venom factors (Knight, L.C. et al. *J. Nucl. Med.* 1994, 35, 282-288), anti-platelet factor IV (Pearson, D.A. et al. *J. Med. Chem.* 1996, 39, 1372-1382), and the DuPont peptide DMP444 (Line, B.R. et al. *J. Nucl. Med.* 1996, 37, 117P), the peptide, 30 Cys-Ser-Val-Thr-Cys-Gly (SEQ ID NO: 4) is an integral part of a protein that exists naturally in the molecular domain of thrombospondin, and one which appears to play a role in the formation of thrombi via activated platelets.

SUMMARY OF THE INVENTION

35 The present invention provides a composition useful as a radiodiagnostic agent for imaging thrombi, pulmonary emboli,

- 7 -

or lesions from atherosclerosis in a mammals, a method and a kit for the preparation of the thrombus imaging agent, a reagent for radiolabeling the imaging agent, and a method of use for the thrombus imaging agent. Specifically, the
5 thrombus imaging agent is comprised of a composition containing a thrombus specific sequence (TSS) and a radionuclide moiety linked to the TSS via it linker, wherein a radionuclide is complexed to the radionuclide moiety. In a preferred embodiment, the composition comprises the sequence
10 Gly (D)Ala Gly Gly Aba Tyr Cys(Acm) Ser Val Thr Cys(Acm) Gly (SEQ ID NO: 7).

A second embodiment of the invention provides a reagent for radiolabeling a TSS, comprising four amino acids, selected from the group consisting of glycine and alanine, which can
15 covalently link a selected radionuclide to the amino groups of each amino acid to form an N₄ configuration.

Still another embodiment of the invention provides a kit for preparing a thrombus imaging agent, which kit comprises a container capable of holding a multiple of vials and
20 reagents. A first vial contains an appropriate quantity of prepared TSS and radionuclide moiety for reacting with a radionuclide. A second vial contains an appropriate buffer.

The invention also provides a method of imaging vascular thrombi, pulmonary emboli, or lesions from atherosclerosis in
25 a mammal by obtaining *in vivo* gamma scintigraphic images. The method comprises administering an effective diagnostic amount of the thrombus imaging agent to a mammal in need of such imaging and detecting the gamma radiation emitted by the imaging agent localized at the thrombus site within the
30 mammal.

DESCRIPTION OF THE FIGURES

Figure 1 is a structural representation of a thrombus imaging agent.

Figure 2 is a plot of the ion spray mass spectrum of a
35 TSS showing a single mass peak at 1201.4 (expected mass 1201.5).

- 8 -

Figure 3A is a plot of the HPLC elution spectra for a rabbit urine sample obtained 4 hours after the administration of a thrombus imaging agent. The diagonal line shows the percent solvent composition. Figure 3B is a plot of the HPLC 5 elution spectra for a thrombus imaging agent. The free technetium eluted at retention time 3.7 and the bound technetium eluted at retention time 9.3. The diagonal line shows the percent solvent composition.

Figure 4A is the gamma camera images for four separate 10 blood clots that were formed concurrently with the addition of the thrombus imaging agent. The brightness of the spots is proportional to the level of radioactivity. Figure 4B is the gamma camera images for five separate blood clots that were formed prior to the addition of the thrombus imaging 15 agent. The brightness of the spots is proportional to the level of radioactivity. Figure 4C is the gamma camera images for three separate blood clots that were formed following incubation for two hours with the thrombus imaging agent. The brightness of the spots is proportional to the level of 20 radioactivity.

Figure 5A is a venogram of the anterior lower extremities of a rabbit. The arrow indicates the position of a rupture of the right iliac artery. Figure 5B is a venogram 25 of an anterior lower extremities of a rabbit. The arrow indicates the position of a thrombogenic coil in the left iliac artery above the hip joint.

Figure 6 is a gamma camera image of the anterior (lower abdominal) region of a rabbit obtained 15 minutes after the administration of the thrombus imaging agent. The solid arrow 30 indicates an area of high radioactivity concentration corresponding to a thrombus in the vicinity of a ruptured iliac artery. The open arrow indicates a photopenic area near the left hip joint where a thrombogenic coil had lodged and blood flow had ceased due to an air embolus.

35 Figure 7 is a schematic representation of the synthesis of Tc99m-TSS.

- 9 -

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations and Definitions

1. Abbreviations

	Aba	(D)-4-aminobutyric acid
5	ADP	Adenosine 5'-diphosphate
	BSA	Bovine serum albumin
	C (Acm)	Acetaminomethyl blocked cysteine residue
	DVT	Deep venous thrombosis
10	Hepes	N-[2-Hydroxyethyl]piperazine-N' [2-ethanesulfonic acid]
	HPLC	High pressure liquid chromatography
	HSA	Human serum albumin
	KB tumor cells	Human oral carcinoma cells
	KeV	Kiloelectron volt
15	MDA	M.D. Anderson cell line
	PE	Pulmonary embolism
	TFA	Trifluoroacetic acid
	TSP	Thrombospondin, first identified as thrombin sensitive protein
20	TSS	Thrombus specific sequence

2. Definitions

"Natural amino acid" means any of the twenty primary, naturally occurring amino acids which typically form peptides and polypeptides. By "synthetic amino acid" is meant any other amino acid, regardless of whether it is prepared synthetically or derived from a natural source.

"Radiolabeling moiety" is a sequence comprising four amino acids capable of complexing with a selected radionuclide in an N₄ configuration.

30 "Radiolabeled complex" is the complex formed by the radiolabeling moiety and the selected radionuclide.

"Technetium" refers to the radioactive form of technetium, for example, technetium-99m (Tc-99m).

35 "Thrombus imaging agent" refers to a radiolabeled TSS to be used as a radiodiagnostic agent.

"Thrombus specific sequence ("TSS")" refers to a peptide with the sequence, Cys Ser Val Thr Cys Gly (SEQ ID NO: 4) or

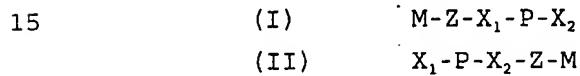
- 10 -

an analog or fragment thereof, to which one or more natural or synthetic amino acids and a linker have been added to the NH₂ or COOH termini.

Thrombus Imaging Agents

5 This invention is directed to a radiodiagnostic agent to image thrombi in a mammal. The invention provides a composition useful as a thrombus imaging agent, a method and kit for preparing the thrombus imaging agent, and a radiolabeling reagent for preparing the thrombus imaging 10 agent. Thrombi as described herein include arterial and venous thrombi, pulmonary emboli, as well as lesions from atherosclerosis..

The thrombus imaging agent is a composition for imaging thrombi in a mammal having the formula of I or II:



wherein:

M is a radiolabeling moiety comprised of four amino acids capable of complexing with a selected radionuclide in 20 an N₄ configuration;

Z is a linker comprised of one or more natural or synthetic amino acids;

X₁ and X₂ are from zero to twenty natural or synthetic amino acids; and

25 P is a peptide comprising the sequence Cys Ser Val Thr Cys Gly (SEQ ID NO: 4), or an analog or fragment thereof, wherein a radionuclide is complexed to the radiolabeling moiety, M of the composition of formula (I) or (II). In a preferred embodiment the composition comprises Gly (D)Ala Gly 30 Gly Aba Tyr Cys(Acm) Ser Val Thr Cys(Acm) Gly (SEQ ID NO: 7) or analog or fragment thereof. Other examples of such analogs include, but are not limited to, the sequences Gly (D)Ala Gly Gly Aba Tyr Cys Ser Val Thr Cys Arg (SEQ ID NO: 8); Gly (D)Ala Gly Gly Aba Tyr Cys Ser Thr Ser Cys Arg (SEQ ID NO: 9); and 35 Gly (D)Ala Gly Gly Aba Tyr Cys Arg Val Thr Cys Gly (SEQ ID NO: 10).

- 11 -

The thrombus imaging agent is comprised of a TSS and a radiolabeled complex connected by the linker of the TSS. By "TSS" it is meant a peptide with the sequence, Cys Ser Val Thr Cys Gly (SEQ ID NO: 4) or an analog or fragment thereof, to which one or more natural or synthetic amino acids and a linker have been added to the NH₂ and/or COOH termini. The linker comprises one or more natural or synthetic amino acids that will preserve the biological activity of the peptide sequence from any steric hindrance imparted by the use of the radionuclide moiety. Preferably, the TSS is comprised of from about 6 to about 30 amino acids, more preferably from about 6 to about 20 amino acids, and most preferably, from about 6 to about 15 amino acids.

In a preferred embodiment of the invention, the cysteines of the TSS have been modified by adding an acetaminomethyl (Acm) blocked cysteine residue to prevent dimerization or cross polymerization, via oxidation, of the two cysteine residues of the TSS. In a preferred embodiment one amino acid, tyrosine (Tyr), and the linker, (D)-4-aminobutyric acid (Aba), have been added to the NH₂ terminus of a sequence, such as SEQ ID NO: 4. Alternatively, an amino acid and a linker could be added to the COOH terminus.

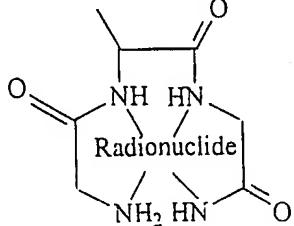
Analogs of the peptide of the TSS can also be utilized in the invention. By "analog" is meant a derivative or modification of the native sequence. For example, the hexapeptide analog Cys Ser Val Thr Cys Arg (SEQ ID NO: 12) has also been demonstrated to be effectively labeled with Tc-99m via a radiolabeling moiety and linker without alteration of its biological activity and to be useful in imaging DVT and PE. One skilled in the art may prepare such analogs wherein the native sequence is modified by resultant single or multiple amino acid substitutions, additions or deletions. All such modifications resulting in a derivative TSS are included within the scope of the invention, provided that the molecule binds to thrombi and thereby, act as a thrombus imaging agent when radiolabeled.

- 12 -

Conservative amino acid changes may be made which do not alter the biological function of the native sequence. For instance, one polar amino acid, such as threonine, may be substituted for another polar amino acid, such as serine; or 5 one acidic amino acid, such as aspartic acid, may be substituted for another acidic amino acid, such as glutamic acid; or a basic amino acid, such as lysine, arginine or histidine, may be substituted for another basic amino acid; or a non-polar amino acid, such as alanine, leucine or 10 isoleucine, may be substituted for another non-polar amino acid.

Accordingly, an analog as described herein corresponds not only to the native peptide described herein, but also to any analog or fragment thereof which retains the essential 15 biological activity of the peptide. Analogs include any peptide having an amino acid sequence substantially similar to that of SEQ ID NO: 4 in which one or more amino acids have been substituted or inserted in the native sequence. Fragments include peptides of the length less than the full 20 length of SEQ ID NO: 4. The practice of the present invention is, thus, not limited to sequences of the same length as SEQ ID NO: 4, but also includes such fragments of this TSS, provided they bind to thrombi when complexed with the radiolabel moiety and thus, act when radiolabeled as a 25 thrombus imaging agent. Whether an analog or fragment retains the biological activity of the native TSS may be determined by those skilled in the art by following the experimental protocols set forth in herein.

Another embodiment of the invention is the preparation 30 of a radiolabeling moiety for use as a reagent in the production of a thrombus imaging agent. The radiolabeling moiety is capable of complexing with a radionuclide in an N₄ configuration. An N₄ configuration is one having the following structure:



- 13 -

in which the radionuclide is linked to each amino acid through its NH₂ group. While the N₄ configuration is a preferred embodiment for the present invention, other configurations are included within the practice of the present invention. Thus, 5 radiolabeled complexes in which a radionuclide such as technetium is joined to a single thiol moiety or two amino and two thiol moieties (N₂S₂ configuration) are equivalent to the N₄ configuration.

For the preparation of the radiolabeled complex as 10 described herein, four amino acids (the radiolabeling moiety) are covalently linked (or complexed) to a selected radionuclide. Preferably, the amino acids are independently selected from the group consisting of glycine and alanine, provided that at least three of the amino acids are glycine. 15 Alternatively, any combination of amino acids that form an N₄ configuration can be utilized within the scope of this invention. Amino acids of both the D and L enantiomeric configuration can be employed herein. Naturally occurring or synthetic amino acids can be employed herein. In a preferred 20 embodiment, the sequence for the radiolabeling moiety is Gly (D)Ala Gly Gly (SEQ ID NO: 11),

In a preferred embodiment technetium-99m is selected as the radionuclide. However, examples of other suitable radionuclides which can be complexed with this moiety include, 25 but are not limited to, Re-186, Re-188, In-111, Ga-67, Ga-68, Tl-201, Fe-52, Pb-203, Co-58, Cu-64, I-123, I-124, I-125, I-131, At-210, Br-76, Br-77 and F-18.

Another embodiment of the invention is the use of the thrombus imaging agent to image thrombi in mammals, preferably 30 humans. A protocol for such use is provided in Example 6. Thrombus imaging agents of the invention are administered to a mammal in need of such imaging, i.e., suspected of having a thrombi, by intravenous injection. The thrombus imaging agent is administered in a single unit injectable dose at a 35 concentration which is effective for diagnostic purposes. The thrombus imaging agent is administered intravenously in any conventional medium, such as isotonic saline, blood plasma,

- 14 -

or biologically compatible isotonic buffers, such as phosphate, Hepes or Tyrode's buffer. Generally, the unit dose to be administered has a radioactivity of about 0.01 to about 100 mCi, preferably about 1 to 40 mCi. The solution amount 5 to be injected as a unit dose is from about 0.1 ml to about 50.0 ml. Preferably, the amount injected is from about 0.5 to about 5 ml. Imaging of the thrombi can take place within a few minutes of injection. However, imaging can take place, if desired, several hours after injection. In most instances, 10 a sufficient amount of the administered dose will accumulate in the desired area within a few minutes to a few hours after injection to permit the taking of scintigraphy images. This is an "effective diagnostic amount". Any conventional method of scintigraphic imaging, planar, SPECT or PET, for diagnostic 15 purposes, can be utilized in accordance with this invention.

Still another embodiment of the invention is a kit for the preparation of the thrombus imaging agent. An example of such a kit is provided in Example 7. The kit includes a carrier for holding the kit components and containers of the 20 TSS, reducing agent and buffer.

The methods for making and using the thrombus imaging agent of the invention are more fully illustrated in the following examples. These examples illustrate certain aspects of the above-described invention and are shown by way of 25 illumination and not by way of limitation.

EXAMPLES

Example 1: Preparation of Thrombus Imaging Agent

The thrombus imaging agent of the invention described herein was prepared according to the following protocols.

30 A. Preparation of Composition of Formula (I) or (II)

The composition corresponding to the peptide Gly (D)Ala Gly Gly Aba Tyr Cys Ser Val Thr Cys Gly (SEQ ID NO: 7) was prepared on an automated synthesizer (Applied Biosystems Inc., Model No. 430A) using solid phase synthesis with a Wang solid 35 support. The peptide was purified and analyzed by PeptidoGenic Research and Co., Inc. (Livermore, CA). An ion

- 15 -

spray mass spectra analysis of the peptide was conducted on a Perkin Elmer Sciex API-I. An HPLC was performed to purify the peptide using a Shimadz LC-10AD. The column HAISil comprised C18, 5 micron silica. The mass spectrum for a TSS 5 is shown in Figure 2 which indicates a single peak with 98.99% purity having a mass peak of 1201.4 (expected mass 1201.5).

B. Labeling of Thrombus Specific Sequence with Tc-99m.

1. Direct Labeling of Cyclized Peptide with Tc-99m

An initial attempt to prepare a thrombus imaging agent 10 by radiolabeling a peptide by the method of Thakur et al. *Intl. J. Oncol.* 1996, 9, 445-451, in which Tc-99m was covalently bound to sulphhydryl moieties of the cyclized peptide by the reduction of the peptide's cysteine bridge with a 3500 molar excess of sodium ascorbate, was unsuccessful. 15 The cyclized peptide, having the sequence Cys Ser Val Thr Cys Gly (SEQ ID NO: 4) was prepared according to the method of Andreu, D. et al., *Formation of Disulfide Bonds in Synthetic Peptides and Proteins*, Pennington, W., Ed., Humana Press, Totowa, NJ 1994, p. 91-169. One hundred micrograms of the 20 peptide (SEQ ID NO: 4) was dissolved in 1 ml H₂O and the pH adjusted to 7 with NH₄OH. Potassium ferricyanide (2 mg/ml) was added dropwise until a pale yellow color was obtained. The solution was maintained for one hour and then the pH was adjusted to 4.5 with 10% acetic acid. The reaction mixture 25 was passed through a Bio-Rex® 70 column, washed with H₂O and eluted with 50% acetic acid. Fractions (1 ml) were collected and examined by Rainin HPLC using a reverse phase Rainin Microsorb-MV C18 column (4.6 x 250 mm) and 0.1% TFA in H₂O (solvent A) and 0.1% TFA in acetonitrile as solvent B at the 30 rate of 1 ml/minutes at 278 nm. The gradient was from about 20% to 90% B in twenty five minutes. Fractions containing the peptide were lyophilized by the method of Thakur et al., *supra*.

The labeled cyclized peptide was then assayed for its 35 ability to bind to resting and activated platelets. The platelets used in this assay were obtained from healthy human volunteers. Platelets (0.5 ml; 0.7 x 10⁹ platelets), which

- 16 -

were stored in a salt balanced medium for up to four days and 20 μ l of the technetium labeled cyclized peptide (approximately 1.9×10^{-3} mg) were dispensed into each of six test tubes. To activate the platelets, 6 i.u. of thrombin was 5 added to three of the tubes and gently mixed until a visible clot was formed. The remaining three tubes (absent thrombin) served as controls, i.e., resting platelets. The tubes were held at 22°C for 15 minutes and then centrifuged at 2000 g for 10 minutes. The supernatant was separated and the platelets 10 washed with 0.9% NaCl and centrifuged again at 2000 g for 10 minutes. Radioactivity in the supernatant and washings combined, and that associated with the platelet samples, was counted and the percentage of radiolabeled platelets in each tube was determined. The binding of the peptide to the 15 activated platelets was poor (<2%). Without wishing to be bound by any theory, it is believed that the poor binding of the labeled cyclized peptide may have been due, in part, to cross linking of the peptide molecules which resulted in diminished biological activity of the peptide.

20 2. Indirect Labeling of TSS with Tc-99m

To prepare a thrombus imaging agent as described herein, the cysteine residues of a peptide having the sequence Cys Ser Val Thr Cys Gly (SEQ ID NO: 4) were protected by Acm, according to the method of Veber, D., et al. *J. Am. Chem. Soc.* 25 1972, 94, 5456-5461, prior to labeling with Tc-99m to prevent the formation of disulfide bonds between the cysteines. To eliminate any potential steric hindrance from the radiolabeling moiety, i.e., the amino acids, Gly (D)Ala Gly Gly (SEQ ID NO: 11), a linker, Aba, and one amino acid, Tyr, 30 were added to the NH₂ terminus of Cys-1 of the peptide prior to the addition of the radiolabeling moiety amino acids. Without wishing to be bound by any theory, it is believed that the biological activity of the peptide directly labeled with Tc-99m, as described in Section B.1 above, was eliminated, in 35 part, due to steric hindrance imparted by the radiolabeling moiety, or by cross linkage of the peptide. Thus, the composition referred to herein as SEQ ID NO: 7 is comprised

- 17 -

of a peptide (SEQ ID NO: 4), a radiolabeling moiety (SEQ ID NO: 11) and a linker, and may also include one or more amino acids.

Technetium-99m labeling of the composition was carried 5 out according to the method as depicted in Figure 7.

One hundred μ g of the prepared composition (1 mg/ml 0.9% NaCl) was lyophilized in the presence of 50 μ g SnCl₂ (10 μ l, 0.05 M HCl) and 100 μ l 0.05 M acetate buffer at pH 4.2. The vials were frozen in a dry ice/acetone bath and lyophilized 10 in a CVP 100/2 GeneVac (GeneVac Ltd., Manchester, England) lyophilizer for two hours. The vials were sealed under nitrogen and stored at 4°C. At the time of radiolabeling, 500 μ l of 0.1 M Na₃PO₄ solution (pH 12) was added to the vial, followed by 5-40 mCi of Tc-99m solution in 0.3-0.5 ml 0.9% 15 NaCl. The content was vortexed and incubated at 22°C (room temperature) for 15 minutes. One and a half ml of 0.1 M Na H₂PO₄ (pH 4.6) was added to the reaction mixture to adjust the mixture to a final pH of 6.0-6.5.

After 15 minutes incubation, greater than 95% of the 20 radioactive agent was incorporated. If desired, unbound Tc-99m can be eliminated by passing the reaction mixture through a C-18 Sep Pak cartridge (Waters Associates, Boston, MA), as described in Thakur, M. et al. *Intl. J. Oncol.* 1996, 9, 445-451. The radiolabeled composition was eluted in 2 ml 95% 25 ethanol, which was then evaporated to dryness and the residue taken up in a suitable volume of 0.9% NaCl.

The purity of the product was examined by HPLC (Rainin, Emeryville, CA) using a reverse phase C-18 column and gradient solvents of 0.1% TFA in water (solvent A) and 0.1% TFA in 30 acetonitrile (solvent B). The gradient was such that at zero minutes, solvent A was 30%, reaching to solvent V 100% in 30 minutes. The flow rate was 1 ml/minute. The HPLC was equipped with a u.v. detector set at 278 nm, a 2" NaI (Tl) gamma counter and a rate meter. The radiolabeled composition 35 (SEQ ID NO: 7), the thrombus imaging agent, was eluted at retention time 9.3 minutes, and the unbounded technetium at

- 18 -

3.7 minutes. As shown in Figure 3B, free radioactivity was less than 2%.

The *in vitro* stability of the thrombus imaging agent was examined for up to 18 hours after preparation. Periodic HPLC analyses showed that the quantity of free Tc-99m had not increased from the percentage of unbound Tc-99m at the time of the preparation.

Alternatively, the stability of the thrombus imaging agent was determined by comparing an HPLC elution plot of the radiolabeled composition (SEQ ID NO: 7) and of the radioactivity excreted in the urine of the rabbit given the thrombus imaging agent four hours previously. For the *in vivo* analysis, a preparation containing the radiolabeled composition (SEQ ID NO: 7) was injected intravenously into a New Zealand white rabbit. Urine was collected from the rabbit at four hours post-injection and a 20 μ l portion of which was analyzed by HPLC as described above. The animal had not voided prior to collection of this sample. As shown in Figure 3A, the quantity of free Tc-99m (at 3.7 minutes) in the urine was substantially the same to that in the free form (Figure 3B). The proportion of bound Tc-99m in the *in vivo* sample was eluted in a single peak, with a retention time of 9.3 minutes, which was substantially the same as that eluted in the thrombus imaging agent.

25 Example 2: Cell Adhesion Of Thrombus Imaging Agent

The following assay was performed to demonstrate the biological activity of a thrombus specific sequence.

Using the method of Tuszyński et al. *J. Cell Biol.* 1992, 116, 209-221, the hexapeptide Cys Ser Val Thr Cys Gly (SEQ ID NO: 4) and fibrinectin were used as controls, and the composition of SEQ ID NO: 7, as a test peptide. None of the peptides or fibrinectin were radiolabeled. Fifty μ l of a 200 mg/ml saline solution of the peptide or protein was air dried overnight in wells of a microtiter plate. After drying, the wells were blocked with 50 μ l of BSA for 30 minutes and washed with Hepes-buffered saline. The wells were incubated for one

- 19 -

hour with a titer of 3.3×10^4 MDA-231 cells in 100 μl of Hepes-buffered saline containing 5 mM glucose, 1 mM MgCl₂ and 1 mM CaCl₂. The wells were washed once with PBS and adherent cells were fixed with 3% glutaraldehyde and stained with 5 Giemsa overnight. The total number of adherent cells were counted under light microscopy. No statistical difference in the proportion of cells demonstrating cell adhesion was observed between the test peptide (SEQ ID NO: 7) and the hexapeptide (SEQ ID NO: 4). However, approximately 17% lower 10 cell adhesion was observed for the test peptide (SEQ ID NO: 7) and the hexapeptide (SEQ ID NO: 4) as compared to fibronectin.

Example 3: Binding of Thrombus Imaging Agent to Resting and Activated Platelets

15 A thrombus imaging agent, i.e., the composition of SEQ ID NO: 7 labeled with Tc-99m, was assayed for its ability to bind to resting and activated platelets. Again, the platelets used in this assay were obtained from healthy volunteers. Platelets (0.5 ml; 0.7×10^9 platelets), which were stored for 20 up to four days and 20 μl of the thrombus imaging agent ($\sim 1.9 \times 10^{-3}$ μg) were dispensed into each of six test tubes. To activate the platelets, 6 i.u. of thrombin was added to three of the tubes and gently mixed until a visible clot was formed. The remaining three tubes (absent thrombin) served as controls 25 (i.e., resting platelets). The tubes were held at 22°C for 15 minutes and then centrifuged at 2000 g for 10 minutes. The supernatant was separated and the platelets washed with 0.9% NaCl and centrifuged again at 2000 g for 10 minutes. Radioactivity in the supernatant and washings combined, and 30 that associated with the platelet samples, was counted and the percentage of radiolabeled platelets in each tube was determined. Results showed that $2.1 \pm 0.5\%$ of the added radiolabel was bound to resting platelets, as compared to $12.5 \pm 1.2\%$ for the activated platelets.

35 This assay can also be used to screen for biological activity for any thrombus imaging agent.

- 20 -

**Example 4: Binding of the Thrombus Imaging Agent to Forming
and Pre-formed Human Blood Clots In Vitro**

In several 1 ml syringes, approximately 0.7 ml of blood was drawn, without any anti-coagulant, from a healthy human 5 volunteer. In four of the syringes, approximately 2 μ Ci of the thrombus imaging agent was added and mixed, and followed by the addition of 15 i.u. units of thrombin and mixed again. Clots were allowed to form for 15 minutes at 22°C. The fluid 10 was expelled and reserved, and the clots were washed with 0.9% NaCl. The wash was combined with the reserved expelled fluid. The clots, still in the syringe, were placed over a gamma camera (GE 300 STARCAM) and imaged for a combined total count 15 of 35,000. The forming clots were expelled from the syringes, collected in four separate test tubes, and placed in a gamma counter (Packard 5000 series) together with the previously reserved expelled liquid for each respective sample. The percentage of radioactivity associated with each clot was determined.

In the four remaining test tubes, thrombin was added and 20 allowed to incubate for 15 minutes, prior to the addition of the thrombus imaging agent. The pre-formed clots were treated as described above and measured for radioactivity

Images of the forming clots and pre-formed clots are shown in Figures 4A and 4B, which qualitatively illustrate 25 that there was more radioactivity in the forming clots than in the preformed clots. The greater the level of radioactivity, the greater the expectation that the thrombus imaging agent can be used to image thrombi. It is expected that *in vivo*, where activated platelets may continue to 30 accrete on a clot, that the proportion of radioactivity corresponding to activated platelets will be greater. While not wishing to be bound by any theory, it is believed that the proportion of labeled platelets in the preformed clot *in vitro* is somewhat less due to the instance of platelets being 35 already embedded in the clot, analogous to the instance of a pulmonary embolism, leaving fewer activated platelets for the thrombus imaging agent to bind. This may be one reason for

- 21 -

less activity taken up in the preformed clot. Further, again without wishing to be bound by any theory, it is believed that the lack of uniform distribution of radioactivity in the forming clots (Figure 4A) may be due to the difficulty of 5 obtaining a uniform mixture of thrombin in the narrow diameter of a one ml syringe. The forming clots of this assay are analogous to the situation of a venous thrombi. Thus, it has been shown that the thrombus imaging agent as described herein will bind to the forming clot in a blood vessel.

10 A quantitative assay was also carried out that showed that the thrombus imaging agent of the present invention binds to activated platelets. The radioactivity level measured in the forming clot was $27.6 \pm 0.2\%$ as compared to $6.4 \pm 3.2\%$ in the preformed clots.

15 In still another assay to demonstrate the binding ability of the thrombus imaging agent, the thrombus imaging agent was incubated for 2 hours at 22°C with three samples of the forming clots. Images of these (broken) clots are shown in Figure 4C, in which $51 \pm 1.2\%$ of the added radiolabel was 20 taken up. The increasing uptake of the thrombus imaging agent over a prolonged period of time in a forming clot *in vitro* is analogous to the situation in patients in whom clots continue to form slowly for a long period of time.

Example 5: Use of a Thrombus Imaging Agent *In Vivo*

25 The following study demonstrates that a thrombus imaging agent of the invention can be used to image thrombi *in vivo*. An experimental clot was induced in a femoral artery of a rabbit and imaged by scintigraphy.

A New Zealand white albino rabbit (3.5 kg) was 30 anesthetized with a mixture of ketamine/zylaxine and placed in the supine position on an x-ray table. The animal was not heparinized. A small incision was made in the neck to expose the right carotid artery; a catheter inserted and with the assistance of a guidewire and a fluoroscope, the catheter was 35 advanced in the left femoral artery. As shown in Figure 5A, a small puncture from the guidewire accidentally occurred in

- 22 -

the right femoral artery when the catheter was inserted. The puncture was confirmed when contrast agent was injected. A 3 mm x 2 cm thrombogenic coil (Cooks Inc., Bloomington, IN) was placed in the catheter, and with the help of the guidewire 5. was lodged in the left femoral artery above the hip joint, near the bladder (Figure 5B).

Approximately 25 minutes after the insertion of the catheter and coil, 2.4 μ Ci (10 μ g) of the thrombus imaging agent was injected intravenously into the animal and serial 10 gamma camera images were obtained.

Figure 6 shows that an intense uptake of radioactivity occurred at the right lower abdomen where the artery had been punctured, i.e., that a clot had formed, and that a "cold spot," an area where a small amount or no radioactivity had 15 accumulated, occurred where the coil was placed. Without wishing to be bound by any theory, it is presumed that the cold spot is due to the lack of blood flow from an air embolus to the area where the coil was inserted.

The animal was sacrificed 4 hours later and a section 20 of the left femoral artery with the coil in place was retrieved. As is evident in Figure 6, an intense image from the thrombi in the left artery is visible. No clot had formed in the right artery, presumably because of an air embolus which prevented blood flow down to the artery where the coil 25 was lodged. The appearance of the cold spot confirms the absence of blood circulation in this area and supports the air embolus hypothesis.

Example 6: Imaging vascular thrombosis with peptide analog

Experiments were also conducted with a thrombus imaging 30 agent wherein the hexapeptide P is the analog Cys Ser Val Thr Cys Arg (SEQ ID NO: 12). This peptide analog was modified as Gly Ala Gly Gly Aba Cys(Acm) Ser Val Thr Cys(Acm) Arg (TP-1300; SEQ ID NO: 8) to provide an N₄ configuration for chelation with Tc-99m and a spacer that minimized steric 35 hindrance. TP-1300 was then evaluated for its ability to inhibit platelet aggregation and Tc-99m-TP-1300 was examined

- 23 -

for its ability to specifically bind to activated platelets. In rabbits, venous thrombi (DVT) were induced using an intravascular electrode as described by Leadley et al. *Thrombosis and Hemostasis*, 1995, 74, 1583-1590 which alters 5 the intima by 150 μ A current. Pulmonary emboli (PE) were formed by injecting, through a carotid artery, 3 mm x 5.8 mm tantalum impregnated autologous blood clots prepared *in vitro*. Ten minutes to 48 hours later, Tc-99m-TP-1300 was injected 10 intravenously. Serial images were obtained for 4 hours. Animals were then sacrificed; excised lungs were X-rayed; and 15 PE, DVT, blood and other tissues were excised for quantification of radioactivity.

TP-1300 facilitated quantitative incorporation of Tc-99m and inhibited ADP induced rabbit and human platelet 15 aggregation (153 μ M). The uptake of Tc-99m-TP-1300 on thrombin activated rabbit, swine and dog platelets was 3.5, 3.5 and 20 time more than on resting platelets. DVT, which weighed between 1-5 mg, were detectable, giving DVT/blood 20 radioactivity ratios of 2.3-63.5. PE weighed 50-100 mg and PE/blood ratios were 1.2-5.

Example 7: Use of Thrombus Imaging Agent to Image Thrombi in Humans

The following example illustrates the use of a thrombus 25 imaging agent of the present invention to image thrombi in a human patient.

The thrombus imaging agent will be available as a composition of formula (I) or (II) in a lyophilized powder form in a sealed vial. The clinician will add to the 30 composition a sufficient quantity of a radionuclide, preferably Tc-99m, to bind and label the composition to form a thrombus imaging agent. Those skilled in the art can readily determine the quantity of Tc-99m to add based on the patient's age and body weight in kilograms (10-20 mCi Tc-99m 35 for a 70 kg person). After dissolving the composition in the Tc-99m, the solution is incubated for 15 minutes at 22°C to ensure that all of the Tc-99m is bound to the composition.

- 24 -

HPLC, performed as described in Example 1, or instant thin layer chromatography can be carried out to verify the proportion of the Tc-99m that is bound to the composition. Drawing the dose in a sterile one ml syringe, the clinician
5 will inject the dose intravenously into the patient. The dose administered is preferably in the range of 10-20 mCi. Those skilled in the art can adjust the dose to be administered for the patient's weight. After 15 minutes, the patient is
10 scanned with a gamma camera calibrated for the gamma ray energy for the radionuclide administered. For Tc-99m, the gamma ray energy is about 140 KeV. Scanning may include a limb in which a clot is suspected or the entire patient body may be imaged. Tomographic images may also be obtained to better delineate the position of the suspected thrombi. The
15 images obtained will then be recorded in a hard copy format, such as by x-ray or paper, for determination of the exact clot position. Using positron emitting radionuclides such as F-18, Ga-68, Cu-64 or I-124, PET imaging could be performed.

**Example 8: Preparation of a Kit for Preparing a Thrombus
20 Imaging Agent**

A preferred kit formulation is one which will label the composition of formula (I) or (II) instantaneously at room temperature, permit greater than 95% of the added radiolabel to bind to the composition, and have a shelf life longer than
25 six months or longer.

The kit will be comprised of a carrier and a vial of a composition of formula (I) or (II), stannous chloride and buffers. The vial will be capable of holding lyophilized reagents as appropriate and will be sealed under nitrogen.
30 Stabilizing agents or anti-oxidants such as EDTA or ascorbic acid may be added to the reagents to increase the shelf life of a kit. Additional vials may contain appropriate reagents including, but not limited to, a buffer, such as 0.05 M phosphate buffer. Antioxidant agents may also be included to
35 prevent oxidation of Sn²⁺ to Sn⁴⁺. Sn²⁺ is necessary for

- 25 -

reduction of Tc⁷⁺ to lower oxidation states required for chelation.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

- 26 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: M. Thakur, V. Pallela
- (ii) TITLE OF INVENTION: Radiolabeled Thrombus Imaging Agents
- (iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Jane Massey Licata, Esq.
- (B) STREET: 66 E. Main Street
- (C) CITY: Marlton
- (D) STATE: NJ
- (E) COUNTRY: USA
- (F) ZIP: 08053

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE
- (B) COMPUTER: IBM 486
- (C) OPERATING SYSTEM: WINDOWS FOR WORKGROUPS
- (D) SOFTWARE: WORDPERFECT 5.1

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: not assigned
- (B) FILING DATE: herewith
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: 08/858,971
- (B) FILING DATE: 5/20/97

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Jane Massey Licata
- (B) REGISTRATION NUMBER: 32,257
- (C) REFERENCE/DOCKET NUMBER: JEFF-0241

- 27 -

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (609) 779-2400
(B) TELEFAX: (609) 810-1454

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Trp Ser Pro Cys Ser Val Thr Cys Gly

1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Arg Gly Asp Cys Ser Val Thr Cys Gly

1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Tyr Cys Ser Val Thr Cys Gly

1 5

(2) INFORMATION FOR SEQ ID NO: 4:

- 28 -

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Cys Ser Val Thr Cys Gly

1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Ala Asn Lys His Tyr Phe

1 5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Val Cys Thr Gly Ser Cys

1 5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

- 29 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Gly Xaa Gly Gly Xaa Tyr Cys Ser Val Thr Cys Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Gly Xaa Gly Gly Xaa Tyr Cys Ser Val Thr Cys Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Gly Xaa Gly Gly Xaa Tyr Cys Ser Thr Ser Cys Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Gly Xaa Gly Gly Xaa Tyr Cys Arg Val Thr Cys Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO: 11:

- 30 -

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Gly Xaa Gly Gly

1

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Cys Ser Val Thr Cys Arg

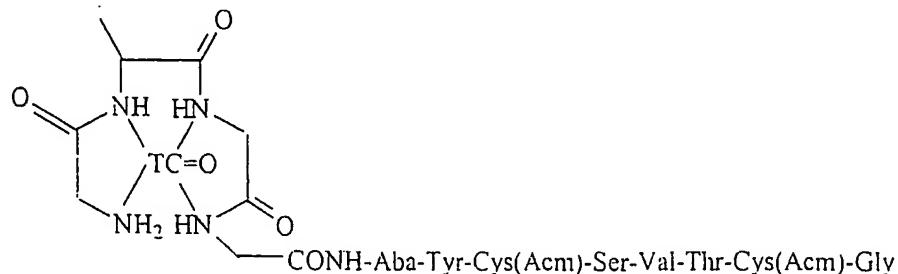
1

5

- 31 -

What is Claimed:

1. A composition having formula I or II:
 - (I) M-Z-X₁-P-X₂
 - (II) X₁-P-X₂-Z-M
- 5 wherein:
 - M is a radiolabeling moiety comprised of four amino acids capable of complexing with a selected radionuclide in an N₄ configuration;
 - Z is a linker comprising one or more natural or 10 synthetic amino acids;
 - X₁ is from zero to twenty natural or synthetic amino acids;
 - P is a peptide comprising a sequence Cys Ser Val Thr Cys Gly (SEQ ID NO: 4), or an analog or fragment thereof; and
 - 15 X₂ is from zero to twenty natural or synthetic amino acids.
2. The composition according to claim 1 comprising SEQ ID NO: 7.
3. The composition according to claim 1 wherein P is an 20 analog comprising SEQ ID NO: 12.
4. The composition according to claim 1 in which the radiolabeling moiety is complexed to the radionuclide.
5. The composition according to claim 4 in which the radionuclide is technetium-99m.
- 25 6. The composition according to claim 4 having the formula:



- 32 -

7. A reagent for radiolabeling a thrombus imaging agent comprising four amino acids which can covalently link a selected radionuclide to the amino group of each amino acid to form an N₄ configuration.

5 8. The reagent according to claim 7 in which the four amino acids are independently selected from the group of glycine and alanine, provided that at least three of the amino acids are glycine.

9. The reagent according to claim 7 in which the four amino acids are Gly (D)Ala Gly Gly (SEQ ID NO: 11).

10. A kit for preparing a thrombus imaging agent comprising:
a carrier for receiving one or more containers;
a first container comprising a sealed vial containing
a predetermined quantity of a thrombi specific sequence; and
15 a second container comprising one or more subcontainers
capable of holding a reducing agent and a buffer.

11. A method for imaging thrombi in a mammal, said method comprising:

injecting into said mammal in need of such imaging an
20 effective diagnostic amount of a composition of claim 4 in a
suitable pharmacological carrier; and

scanning said mammal using radioscintigraphic imaging
apparatus so that any thrombi are imaged.

12. The method according to claim 11 wherein the thrombi to
25 be imaged are venous thrombi.

13. The method according to claim 11 wherein the thrombi to
be imaged are pulmonary emboli.

14. The method according to claim 11 wherein the thrombi to
be imaged are lesions of atherosclerosis.

- 33 -

15. A method for detecting thrombi in a mammal comprising administering an effective diagnostic amount of a composition of claim 4 to a mammal; and detecting the radiolabel localized at the thrombi.

5 16. The method according to claim 15 wherein the thrombi are venous thrombi.

17. The method according to claim 15 wherein the thrombi are pulmonary emboli.

18. The method according to claim 15 wherein the thrombi are
10 lesions of atherosclerosis.

19. A method for preparing a thrombus imaging agent, which method comprises the steps of:

synthesizing a thrombus specific sequence;
linking the thrombus specific sequence to a
15 radiolabeling moiety to produce a composition having formula I or II:

(I) M-Z-X₁-P-X₂

(II) X₁-P-X₂-Z-M

wherein:

20 M is a radiolabeling moiety comprised of four amino acids capable of complexing with a selected radionuclide in an N₄ configuration;

Z is a linker comprising one or more natural or synthetic amino acids;

25 X₁ is from zero to twenty natural or synthetic amino acids;

P is a peptide comprising a sequence Cys Ser Val Thr Cys Gly (SEQ ID NO: 4), or an analog or fragment thereof; and

30 X₂ is from zero to twenty natural or synthetic amino acids; and

combining the composition with a sufficient amount of a selected radiolabel to form a thrombus imaging agent.

- 34 -

20. The method of claim 19 wherein P of the produced composition is an analog comprising SEQ ID NO: 12.

CONH- Aba-Tyr-Cys(Acm)-Ser-Val-Thr-Cys(Acm)-Gly

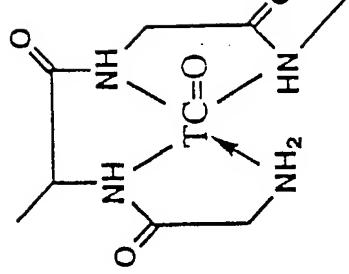
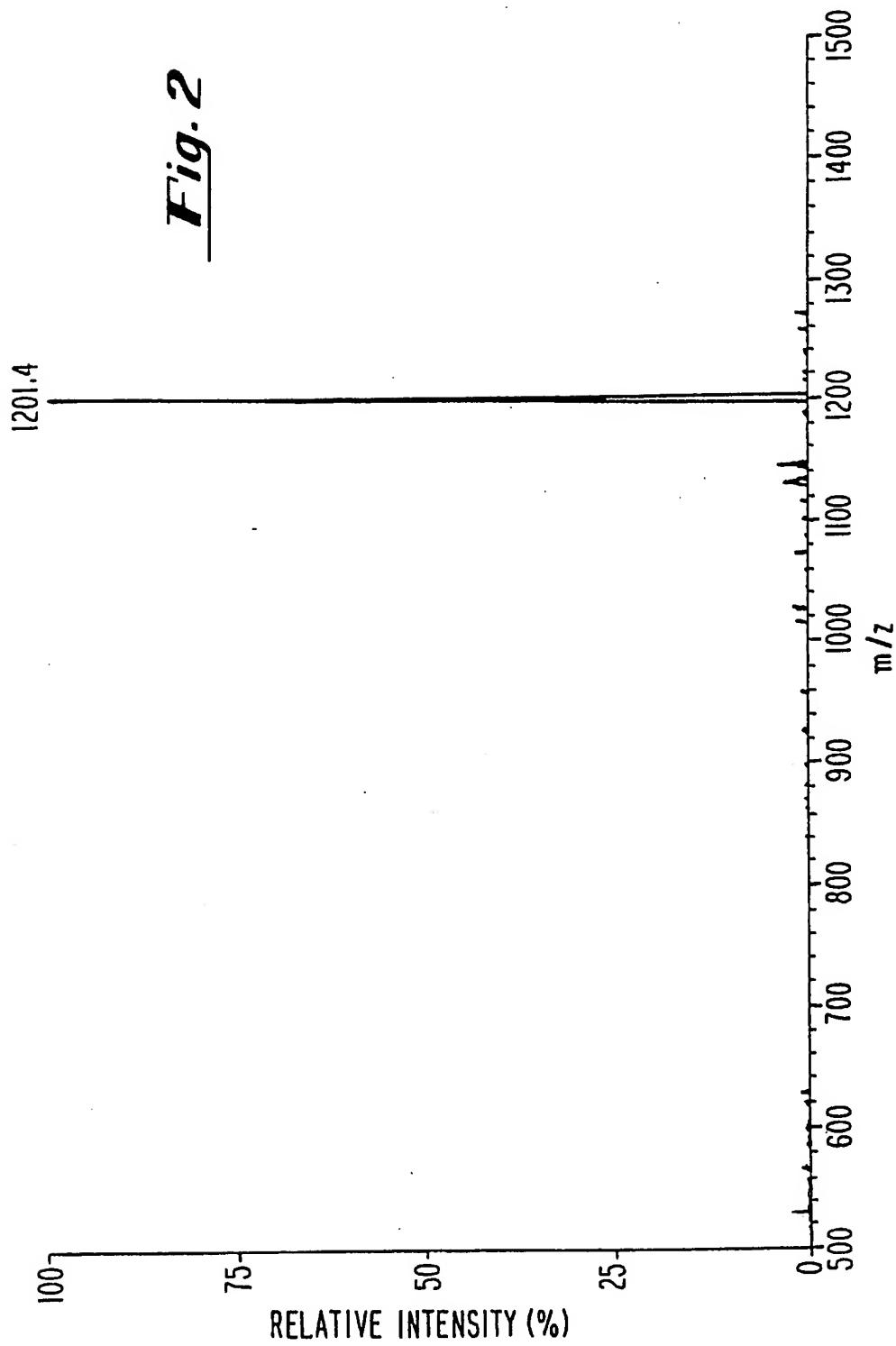


FIG. 1

Fig. 2



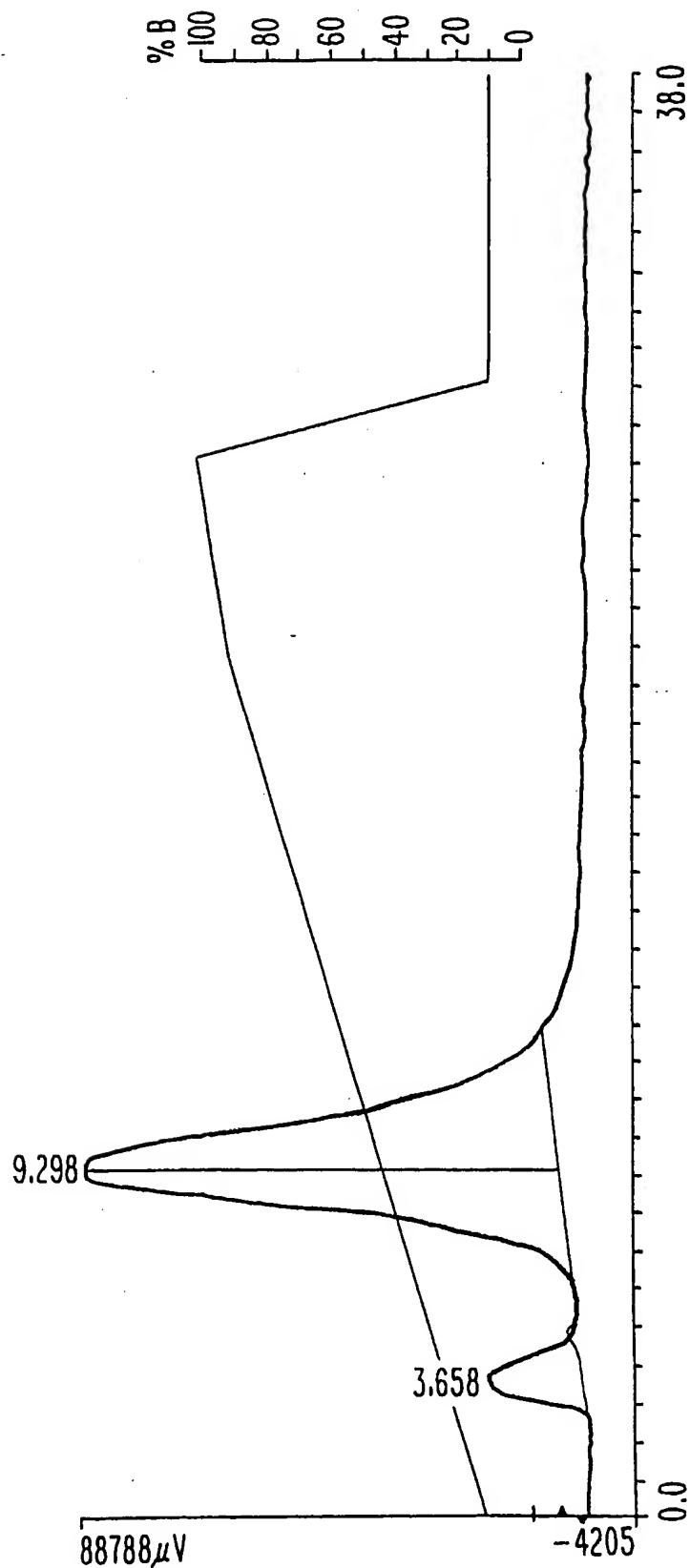


Fig. 3A

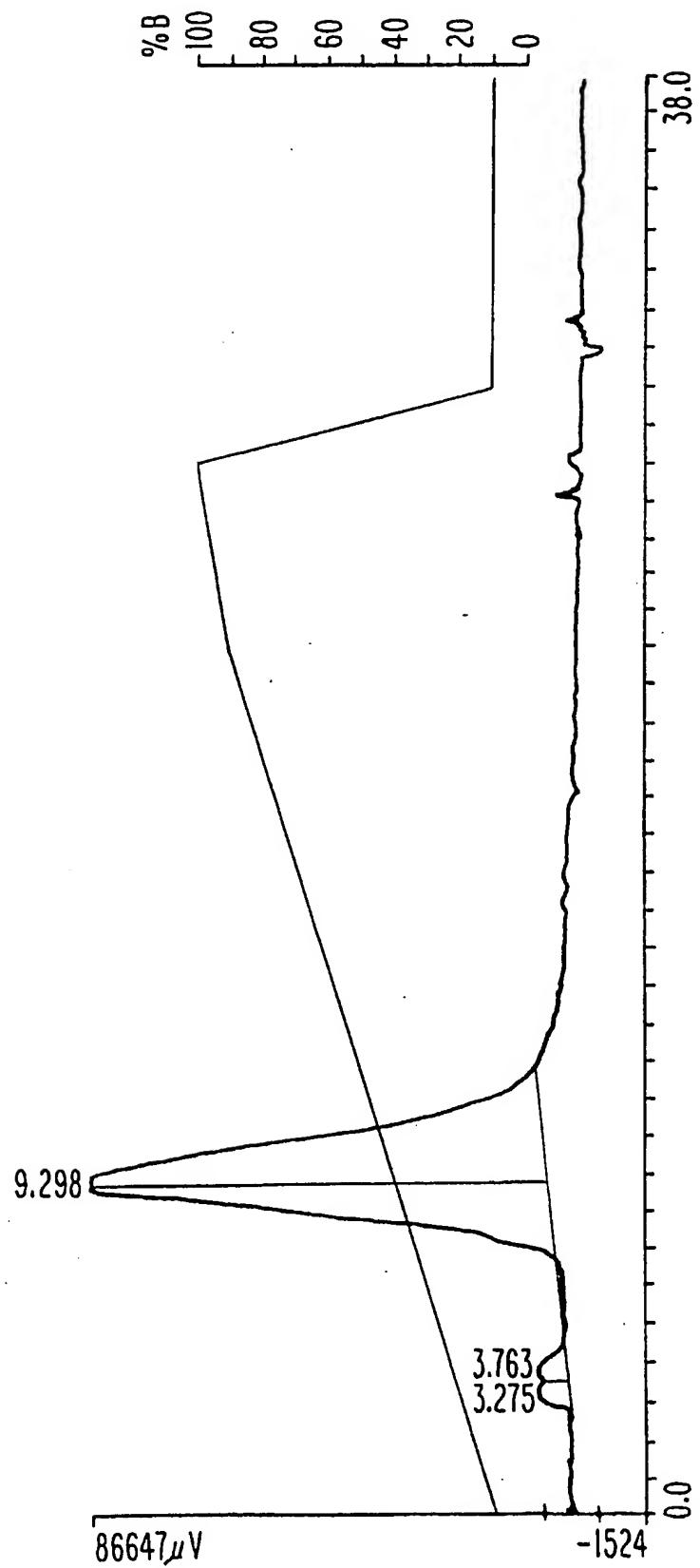


Fig. 3B

5 / 8

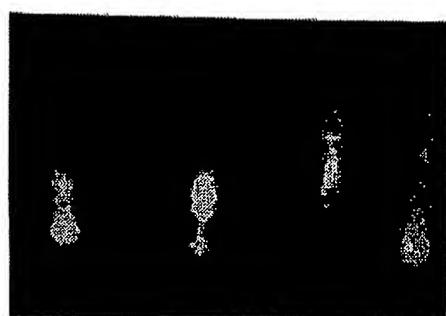


FIG. 4A

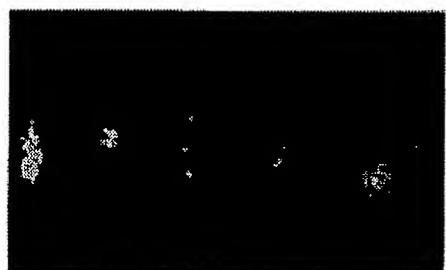


FIG. 4B

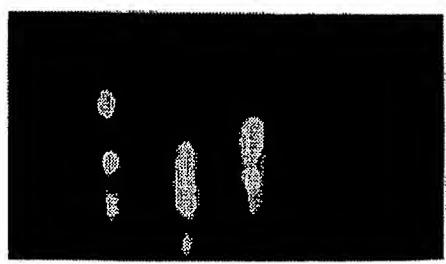


FIG. 4C

6 / 8



FIG. 5A

FIG. 5B

7/8



FIG. 6

Gly-(D)Ala-Gly-Gly-Aba-Tyr-Cys(Acm)-Ser-Val-Thr-Cys(Acm)-Gly

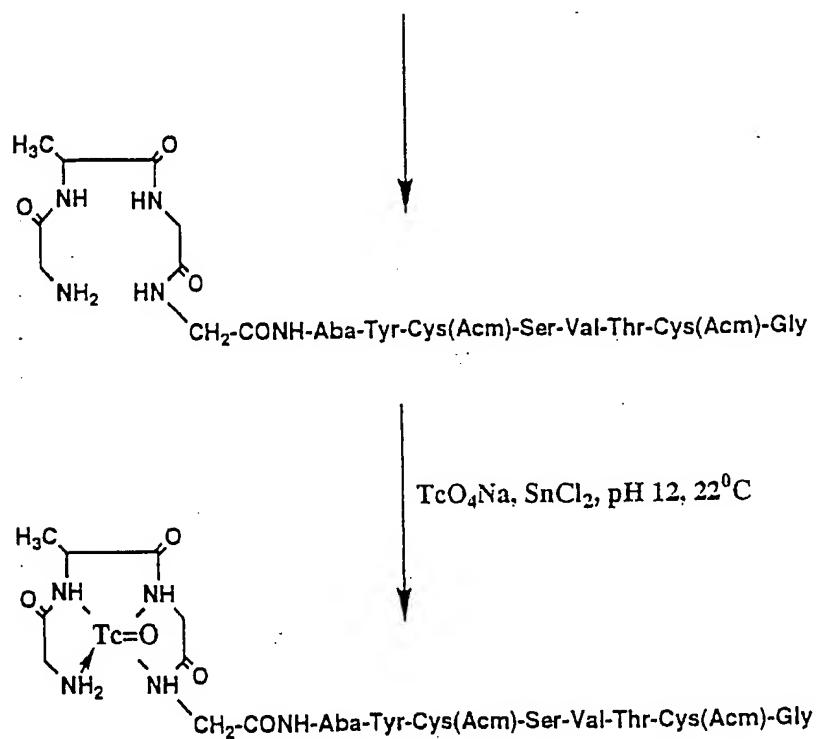


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No. PCT/US98/10186

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 51/00, 38/00; C07K 2/00, 5/00, 7/00; C07F 5/00
US CL :424/1.69, 1.11; 514/12, 14; 530/300, 327, 391.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.69, 1.11; 514/12, 14; 530/300, 327, 391.3.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, BIOSIS, MEDLINE, WPIDS, USPATFULL, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,556,609 A (ZAMORA) 17 September 1996, see entire document.	1-20
Y,P	US 5,736,122 A (DEAN et al) 07 April 1998, see entire document.	1-20
Y,P	US 5,645,815 A (DEAN et al) 08 July 1997, see entire document.	1-20
Y	WO 91/16076 A1 (MALLINCKRODT MEDICAL, INC.) 31 October 1991, see entire document.	1-20

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A"	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search Date of mailing of the international search report

14 SEPTEMBER 1998

14 OCT 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
AVIS M. DAVENPORT
Telephone No. (703) 308-0196